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Immune Responses to *Rickettsia akari* Infection in Congenitally Athymic Nude Mice

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Athymic BALB/c nude mice and euthymic BALB/c mice were infected with *Rickettsia akari* by the intraperitoneal route. The rickettsialpox infection was terminated in euthymic mice with only two intraperitoneal injections of the antibiotic oxytetracycline, whereas prolonged treatment was necessary to terminate the infection in athymic mice. Both athymic and euthymic mice produced specific antibody, but athymic mice were still susceptible to reinfection. Killed *R. akari* served as a protective immunogen in euthymic, but not in athymic, mice. When spleen cells from convalescent euthymic mice were transferred to syngeneic athymic mice, recipients showed protection against challenge. This suggests that a T-cell-dependent step is generally necessary to terminate the rickettsialpox infection.

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Rickettsialpox, caused by *Rickettsia akari*, is a nonfatal spotted fever group disease of humans found in parts of the United States and Russia (6). In contrast to the disease in humans, however, *R. akari* produces a fatal infection in most strains of mice.

The relative roles of humoral and cell-mediated immunity in the elimination of rickettsial infections have not been fully described. Kishimoto et al. (12) showed that *Coxiella burnetii* produced a chronic infection in athymic nude mice, whereas euthymic mice successfully cleared the infection in 14 days. These workers demonstrated that although antibody was produced by athymic mice, the presence of specific antibody was not sufficient to terminate infection. Kázar et al. (7) have shown that resistance to Q fever infection was transferred to syngeneic mice when the animals were given spleen cells from convalescent mice. Similar studies have been reported for scrub typhus rickettsiae (18), but not for spotted fever rickettsiae.

Studies have shown that *Rickettsia rickettsii*, another member of the spotted fever group, is not inactivated with specific antiserum (11). However, other studies with guinea pigs have shown that antibody-coated *R. rickettsii* organisms are killed in vivo (17), probably in macrophages, and such organisms are likewise killed in macrophages grown in vitro (9).

Although rudimentary thymus cells have been found in athymic mice (13) and T-lineage lymphocytes have been found in the spleen (5), these mice have been shown to be devoid of the functional T-lymphocytes required for expression of cell-mediated immunity (15, 16). Therefore, athymic mice are a useful tool for examining the roles of cell-mediated and humoral immunity in defense against infections.

The studies presented here were performed with athymic and syngeneic euthymic mice to examine further the relative importance of humoral and cell-mediated immunity in host defenses against *R. akari* infection.

MATERIALS AND METHODS

Animals. Male inbred BALB/c athymic mice, weighing 18 to 22 g, were obtained from Sprague-Dawley (Madison, Wis.). BALB/c euthymic male mice, also weighing 18 to 22 g, were obtained from Jackson Laboratories (Bar Harbor, Maine). The athymic mice were housed in filter-top cages. Food (Purina 5010; Ralson Purina Co., St. Louis, Mo.), bedding cages, and water acidified to pH 2.5 were sterilized before use.

Rickettsiae. The Kaplan strain of *R. akari* was grown in yolk sacs of embryonated chicken eggs as described previously (8). Rickettsiae were titrated by plaquing as described by Weinberg et al. (20).

Serology. Sera were titrated for *R. akari* antibody by indirect fluorescence microscopy as described by Kenyon et al. (10).

Vaccine. *R. akari* was grown and harvested from chicken embryo cell cultures in the manner described previously for *R. rickettsii* (8). Rickettsiae were partially purified by differential centrifugation and killed with 0.1% Formalin. A total rickettsial count was determined by the method of Silberman and Fiset (19), and the vaccine was adjusted to 5×10^8 rickettsiae per ml. Mice were immunized intraperitoneally (i.p.) by two 0.5-ml injections 2 weeks apart and challenged i.p.

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2 weeks after the second injection with 10^4 plaque-forming units of the *R. akari*.

Antibiotic treatment. Since *R. akari* infection is fatal to both euthymic and athymic mice, infection was aborted by i.p. treatment with 1.25 mg of oxytetracycline per day. Euthymic mice were treated on days 3 and 4 postinfection, and athymic mice were treated on days 3, 4, 6, 9, and 11 postinfection.

Specific antiserum. For studies with *R. akari* antiserum, euthymic mice were infected with 10^4 plaque-forming units of rickettsiae and treated with antibiotic as described above. On day 21, mice were exsanguinated and the serum was sterilized by filtration.

Spleen cell transfer. Preliminary unpublished data from this laboratory with euthymic mice showed that optimum protection against challenge could be conferred by spleen homogenates transferred 14 or 21 days after donor infection. On day 14 postinfection, donor mice were killed by cervical dislocation; spleens were removed aseptically, minced with sterile scalpels, and pressed through sterile 60-mesh stainless-steel screens (Small Parts Co., Miami, Fla.) into cold RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 100 U of penicillin and 100 mg of streptomycin per ml. Cell clumps were dispersed by gentle agitation, and the suspensions were centrifuged at 250 × *g* for 10 min at 5°C, washed three times with antibiotic-free RPMI 1640, and suspended in the same menstruum. Spleen cell suspensions contained at least 95% viable cells as determined by 0.5% trypan blue exclusion. Spleen cells were counted with a Coulter Counter, and the volume was adjusted to contain approximately 5×10^8 spleen cells per ml (equivalent to about 1.2 spleens). Recipients were injected i.p. with 1 ml of spleen cell suspension and then challenged i.p. 16 h later with approximately 10^4 plaque-forming units of *R. akari*. No viable rickettsiae could be isolated from spleens used in adoptive transfer studies. No rickettsiae were observed in spleen impressions examined by fluorescence microscopy.

Peritoneal exudate cell cultures. Mice were injected i.p. with 2 ml of sterile mineral oil (Marcol no. 90; Humble Oil and Refining Co., Houston, Tex.) to stimulate peritoneal exudate cells. After 4 days, the peritoneal exudate cells were collected in E-199 medium with 15% fetal calf serum, 200 U of penicillin, and 200 μg of streptomycin per ml. Approximately 10^6 macrophages were cultured on cover slips in Leighton tubes. After incubation for 4 h at 36°C in an atmosphere of 5% CO_2, cells were washed five times with Hanks balanced salt solution; 1 ml of fresh antibiotic-free E-199 medium with 15% fetal calf serum was then added, and cultures were incubated overnight at 37°C in an atmosphere of 5% CO_2. For interaction studies, live rickettsiae diluted in Hanks balanced salt solution were incubated with an equal volume of normal or immune guinea pig serum (microagglutination titer, 1:4,096) for 30 min at room temperature. The rickettsia-serum mixture was then allowed to interact with macrophages (rickettsia/cell ratio, 1:10) for 60 min at 37°C in a humidified 5% CO_2 atmosphere before the inoculum was replaced with fresh E-199 medium with 15% fetal calf serum. At various times the cultured macrophages were washed with Hanks balanced salt solution, and cover slip preparations were fixed in acetone at −70°C and stained for fluorescence microscopy as described previously (10).

RESULTS

Both athymic and euthymic BALB/c mice survived *R. akari* infection after antibiotic treatment. Preliminary studies showed that two treatments (on days 3 and 4) were sufficient for euthymic mice, but prolonged treatment was necessary for the survival of athymic mice. If treatments were continued through day 11, these mice survived. If treatments were discontinued before day 11, the athymic mice died from infection within 5 or 6 days. Both athymic and euthymic mice developed indirect fluorescence microscopy antibody titers by day 14 (athymic = 1:2,048; euthymic = 1:4,096). However, as shown in Table 1, athymic mice were subject to reinfection when rechallenged, and deaths occurred, whereas euthymic mice were completely resistant to rechallenge. Immunization with Formalin-inactivated *R. akari* completely protected euthymic mice, whereas similarly immunized athymic mice showed no protection against challenge.

The effect of passive transfer of *R. akari* immunity by antiserum is shown in Table 2. Antiserum from euthymic mice protected euthymic mice from a fatal *R. akari* infection. However, the same antiserum did not protect athymic mice; conversely, antiserum from athymic mice completely protected euthymic mice.

The effect of transfer of immune spleen cells to syngeneic recipients is shown in Table 3. When spleen cells from euthymic convalescent mice were transferred to syngeneic euthymic mice, 90% of the recipients survived challenge. When samples of the same spleen cell suspension

TABLE 1. *Effect of various treatments on protection of BALB/c mice against R. akari*[a]

Injection group	No. of survivors/total: Euthymic	Athymic
Killed rickettsiae	5/5	0/5
None	0/5	0/5
Live rickettsiae; antibiotic treated; rechallenged 14 days later[b]	4/4	0/4
None; antibiotic treated; challenged 14 days later	0/4	0/4

[a] Mice were challenged i.p. with approximately 10^4 plaque-forming units of *R. akari*.

[b] Antibody titers in euthymic and athymic groups at the time of challenge were 1:4,096 and 1:2,048, respectively.

were transferred to syngeneic athymic mice, 67% of the recipients survived challenge. Spleen cells from normal, uninfected euthymic mice offered no protection against challenge.

Table 4 shows the effect of antibody coating on the ultimate fate of *R. akari* exposed to peritoneal macrophages grown in vitro. Since a low rickettsia/cell ratio was used, no rickettsiae were observed in any cells 5 h after infection, but by 48 and 72 h, a difference between cells infected with normal versus antibody-treated rickettsiae was evident. When rickettsiae were pretreated with specific antiserum, few or no rickettsiae were observed in the macrophages at 5, 48, or 72 h, whereas rickettsial multiplication occurred after pretreatment with normal serum. There was no difference in the way macrophages from euthymic or athymic mice processed rickettsiae.

DISCUSSION

Recovery from *R. akari* infection of athymic nude mide is prolonged and is only accomplished by prolonged repetitive treatment with antibiotic. This is similar to chronic infections described in the same breed of mice for Q fever (12) and for *Listeria monocytogenes* infections (3). *R. akari* infection, however, can be terminated in euthymic mice with two injections of antibiotic. It appears that immune mechanisms play a minimal role in clearing an infection from athymic mice with antibiotic. Such results suggest that in euthymic mice a T-cell-dependent step is necessary to terminate the rickettsialpox infection.

Circulating antibody is produced by athymic and euthymic mice, suggesting that antibody production against *R. akari* is T-cell independent. Antibody produced in athymic mice appeared to be functional, since it protected against challenge when transferred to normal euthymic mice just before challenge.

There is considerable evidence that antiserum alone does not inactivate rickettsiae (4, 10). Earlier studies from this laboratory showed that *R. rickettsii* multiplied in guinea pig macrophages in vitro, but rickettsiae coated with specific antibody were destroyed by the macrophages (11). Studies by various investigators have shown that the innate early resistance of athymic mice appeared to be due to enhanced activity of macrophages of these mice (1). Since passive transfer of functional *R. akari* antibody did not protect athymic mice, the competency of macrophages from athymic mice was examined. As shown in Table 4, non-antibody-coated *R. akari* replicated equally well in macrophages from euthymic and athymic mice. In addition, macrophages from both types of mice destroyed antibody-coated rickettsiae equally well. In vitro, macrophages from athymic mice can completely destroy *R. akari* in the presence of antibody, but clearing of infection in the presence of antibody apparently does not occur in vivo. One possible explanation for this is that macrophages recruited in vivo in response to mineral oil stimulation are activated and, therefore, more capable of killing antibody-coated rickettsiae (2). The

TABLE 2. *Effect of passive transfer of immunity by antiserum on protection against R. akari*[a]

Donor mouse serum (IFA[b] titer)	Recipient mice[c]	No. survivors/total
Euthymic, convalescent (1:4,096)	Euthymic	4/4
	Athymic	0/4
Athymic, convalescent (1:2,048)	Euthymic	4/4
Euthymic, normal (1:4)	Athymic	0/4

[a] Recipients were challenged i.p. at 4 h with 10^4 plaque-forming units.

[b] IFA, Indirect fluorescent antibody.

[c] Mice were inoculated i.p. with 0.7 ml of antiserum.

TABLE 3. *Effect of spleen cell transfer on immunity to R. akari*[a]

Mouse spleen cell donor	Spleen cell recipient mouse	No. of survivors/total
Convalescent euthymic	Euthymic	9/10
	Athymic	10/15
Normal euthymic	Euthymic	1/5
	Athymic	0/5

[a] Recipients were challenged at 16 h with 10^4 plaque-forming units of *R. akari*.

TABLE 4. *Effect of antibody coating of R. akari on infection of peritoneal macrophages grown in vitro*

Serum treatment	Time postinfection (h)	Cells infected[a] (%)	
		Euthymic mice	Athymic mice
Normal	5	2	1
	48	75	85
	96	99	97
Immune	5	0	0
	48	1	1
	96	1	1
None	5	2	2
	48	80	72
	96	72	99

[a] Cells were examined for rickettsiae by direct fluorescent antibody; four fields of 100 cells were counted to calculate the percent infected.

capability of antiserum from athymic mice to restrict the growth of *R. akari* in macrophages in vitro was not monitored. However, such antiserum apparently was capable of preparing rickettsiae for destruction in vivo, since the transfer of antiserum to euthymic mice protected them from death (Table 1). A successful response to rickettsial infection in vivo may require a T-cell-dependent macrophage activation step which does not occur in athymic mice. However, when immunocompetent spleen cells from convalescent syngeneic donors were introduced, the athymic recipient became refractory to challenge. It seems reasonable that once competent T-cells are available in vivo, potentially competent macrophages in athymic mice will somehow receive the message to activate and destroy invading rickettsiae. Similar results have been reported for another obligate intracellular parasite, *L. monocytogenes*, by Newborg and North (14). They showed that specifically sensitized T-cells are required for the adequate activation of mononuclear phagocytes to eliminate the *Listeria* organisms.

R. akari causes a fatal infection of both athymic and euthymic BALB/c mice. The lack of functional T-cells and the production of functional antibody in athymic mice make these mice an excellent model for the study of acquired resistance to *R. akari*. This model may also be valuable for the study of acquired resistance to other rickettsial infections.

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